Immunotoxicology in the Pharmaceutical Industry

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Development of an immunotoxicology program within the pharmaceutical industry is described. With few guidelines in this area and a multitude of factors to consider, a basic screen for evaluating immune competence in species routinely used in toxicologic studies has been proposed. The future of immunotoxicology depends upon the ability of the selected immune function tests to be predictive of human risk.

The use of immunologic methods over the past several decades has been important in the development of anti-inflammatory drugs. However, the application of immunology within a toxicology environment has only occurred relatively recently. The Immunotoxicology Program at Merck Sharp & Dohme Research Laboratories (MSDRL) has originated within the Department of Safety Assessment. I would like to review the development of this program since its inception three years ago. Since our program is still in its infancy, I must emphasize this paper represents many of my opinions based on the state-of-the-art and future application of immunotoxicology as I perceive them. Because there remains a considerable amount of characterization and refinement of our immune function tests, we do not intend to test new drug candidates until the utility of the procedure is proven.

Historical Perspective

In 1977 there were few guidelines regarding immunotoxicologic testing. The European Economic Community (EEC) drafted the following purpose of immune function tests in the fall of that year: (1) to evaluate the potential risk of the drug by determining the functional significance of the effect

on lymphoid organs found in routine toxicity studies; (2) to obtain more insight into the mode of action (i.e., which cell type is the target and which phase of the immune response is impaired).

At first, this may seem to be a reasonable attempt to acknowledge the importance of immunologic testing, but I call to your attention one particular paragraph in these proposed guidelines:

> At the termination of a toxicity study, thymus, spleen, and lymph nodes should be weighed and examined microscopically. From these data and measurements of serum immunoglobulin fractions and counts of circulating lymphocytes, the conclusion should be reached whether the substance has an effect on the lymphoid system and whether or not specific function tests have to be performed.

The intent, then, is for immune function tests to be done only after histopathologic and hematologic studies have been performed. There are some important pitfalls to this approach. The first is that histopathologic changes are not a sensitive indicator of drug-induced immunologic toxicity. Generally, histomorphologic changes associated with compromised immune function are seen only at fairly high dosage levels. In addition, the inability to differentiate morphologically between T-cells and B-cells is well known. Serum immunoglobulin fractions are equally insensitive as indicators of immune function. They represent an immune response that occurred early in life and are not representative of immune competence in older individuals. The circulating leukocyte count and differential, while

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admittedly important in determining immune competence, could be misleading if absolute numbers are within the normal range. The function of these cells might still be impaired.

None of these tests, with the possible exception of serum immunoglobulin levels, really measures immune function. Yet the EEC guidelines did recommend that certain immune function tests be done, patterned after the review by Vos (1). The scope of these assays involves *in vivo* and *in vitro* testing of cell-mediated immunity (CMI), humoral immunity (HI) and phagocytosis by macrophages. As we shall see later, this approach needs to be tempered to meet the needs of conventional toxicity studies.

Factors That Influence Immunotoxicological Testing

A standard operating procedure in toxicology includes total leukocyte counts and differential, measurement of organ weights (such as adrenal and spleen), and histologic examination of lymphoid organs (spleen, thymus, lymph nodes, and bone marrow). Certain immune function tests would then have to be added to the conventional study in order to make a valid interpretation of immunotoxicologic effects. The panel discussion at the Gordon Conference on Toxicology and Safety Evaluations in August of 1978 did a great deal to prioritize the kinds of determinations that need to be done and which function tests are appropriate to do. On the other hand, some laboratories have proposed a more extensive and perhaps idealistic approach. MSDRL has elected to pursue an approach which applies the most current knowledge of immunology in a practical way.

There are many considerations which tend to complicate an analysis of immune competence. Dosage level and frequency are important variables to consider. Low dosage levels may enhance an immune response whereas higher dosage levels may be immunosuppressive. Whether a drug is administered as a single or multiple dose, as well as the route of administration, will contribute to the effect observed. Furthermore, it is important to correlate immune function tests with the dosage regimens used. All too frequently, substantially different protocols are used to measure CMI and HI, with particular reference to the time of administration of the test compound with respect to the time the immunologic stimulus (antigen) is introduced. Consequently, any differences between immune responses are not easy to interpret. Finally, the age of the animals being tested is particu-

larly critical from an immunologic standpoint. The prenatal period, early postnatal period and late adulthood may be especially vulnerable to immunologic alterations. In fact, there does appear to be a natural loss of immunoreactivity with age (2).

Another factor would be the species employed for immunologic testing, taking into consideration drug metabolism. For example, the difference in the LD_{50} between mice and rats for cyclophosphamide (3) most likely represents differences in metabolism to the reactive intermediate. One fundamental difference between routine toxicologic evaluation and basic research in immunology is the use of random-bred versus inbred strains, respectively. Random-bred strains may accentuate animal-to-animal variation, especially since many immune responses are under genetic control (4). The adaptation of universally accepted assays in one species (i.e., mouse) to a species typically used in toxicology (e.g., rat) requires adequate characterization. For instance, one should not assume that a B-cell mitogen (lipopolysaccharide) in the mouse is automatically a B-cell mitogen in the rat.

Secondary factors resulting from stress-related phenomena (i.e., adrenocortical hypertrophy) can also lead to immunologic changes. Other important considerations include the relative sensitivity of immunologic assays, method of calculation, correlation of *in vivo* and *in vitro* findings, differences between sexes, production of delayed or biphasic immunobiologic responses, presence of contaminants in the feed or drinking water and the length of the study.

The ultimate proof of an immunologic evaluation is the ability to predict human risk. One of our most frequently asked questions is: what does it mean if the hematologic, serum biochemical and gross and histopathologic examinations show no effect due to treatment, but an immune function test indicates an alteration? The answer, of course, depends upon the predictability of the immune function test. A more pragmatic approach might be to ask whether a given compound will cause hypogammaglobulinemia, alter the state of hypersensitivity to pollens, food or contact allergens, increase susceptibility to infection, alter complement activity or induce an autoimmune reaction, all of which are highly relevant questions regarding human risk. Indeed, determination of serum immunoglobulin levels, induction of delayed-type hypersensitivity reactions in skin, quantitation of specific antibody titers, determination of lymphoproliferative potential, and enumeration of T- and B-cells form the basis of clinical immunology.

It is important to recognize that the evaluation

of immune competence is related to immune responsiveness and is not simply the examination of lymphoid tissue at a single point in time using histopathologic techniques and hematology. Four steps of the immune response that may be altered by drugs are: (a) the recognition of antigen, (b) clonal proliferation, (c) differentiation and (d) the effector phase, characterized by the infiltration of immune cells into the site of an immunologic reaction and the secretion of lymphokines, induction of delayed hypersensitivity, graft rejection, and/or elaboration of specific antibodies. Drugs that block contractile microfilaments, such as cytochalasin B, might be expected to interfere with the antigen processing by macrophages. The majority of antineoplastic drugs are known to interrupt the proliferative phase of lymphocytes by blocking DNA synthesis.

In summary, the scope of any immunotoxicology program should be to develop tests that can reliably, efficiently, and inexpensively screen a large number of compounds for their ability to modify the immune response. Because of the complexity of the immune system, a panel of tests, both *in vitro* and *in vivo* must be used. Testing should proceed in basically two phases: simple, straightforward assays that are capable of detecting major alterations in the immune response (such as antibody production to a T-dependent antigen which requires helper T-cells, macrophages, and B-cells) followed by more complicated procedures (specific immune models) that elucidate mechanisms of drug actions and identify cell types.

Development of an Immunotoxicology Program

At MSDRL we started with the assumption that the evaluation of immune competence would be performed during a routine toxicity study. This approach offers several advantages, including the coordination of immunologic tests with other toxicological parameters (i.e., hemotology, serum biochemistry, and histopathology), collection of multiple blood samples, potential to build a large historical data base and detection of novel immune modulators that would not have otherwise gone through an immunologic screen. However, before immunotoxicology can begin to pay dividends, progress will need to be made in some areas, such as increasing our understanding of the mechanisms involved in the immune response, adapting immunologic methods from one species to another to obtain interspecies correlation of data, arriving at a consensus on which assays should be performed

and accepting a multifaceted approach to delineate the mechanism of drug action (not necessarily in that order).

Originally, three fundamental immunologic function areas were designated. The primary objectives were to select tests that could be done in rats, dogs, and monkeys and would interface with the standard operating procedures of our laboratory, such as the collection of blood samples at the same time as routine bleedings for hematologic and serum biochemical studies.

Serum Immunoglobulin Levels

The quantitation of serum immunoglobulin levels, specifically IgG, IgM, and IgA, was chosen as a crude reflection of B-cell activity. Since we usually analyze a relatively large volume of samples in a day, it was apparent that many of the currently available techniques to quantitate specific serum proteins (i.e., radial immunodiffusion, radioimmunoassay, and electroimmunodiffusion) were unsuitable because of lack of automation. Another initial requisite, commercially available reagents, also was not feasible at that time; reagents were available to quantitate human serum immunoglobulins, but not for rats, dogs, and monkeys. We chose to produce monospecific, high avidity antibodies from goats directed against IgG, IgM and IgA from all three species and to use the nephelometric technology developed by Ritchie (5). This highly sensitive method is capable of detecting the microprecipitate formed from an antigen (IgG, IgM, or IgA)–antibody reaction. Other automated systems are becoming available and we are in the process of evaluating them for the purpose of quantitating serum immunoglobulins.

Lymphocyte Blastogenesis

Perhaps one of the most universal assays used in immunology today is the induction of lymphocyte proliferation with mitogens or specific proteins, also known as the transformation or blastogenic assay. Certain plant lectins, such as concanavalin (ConA) and phytohemagglutinin (PHA) selectively stimulate T-cells to undergo DNA synthesis in a polyclonal fashion. On the other hand, bacterial products, such as lipopolysaccharide, polyclonally stimulate B-cells (at least in the mouse). The search for a potent B-cell mitogen in other species is still continuing: *Mycoplasma neurolyticum* appears to be a B-cell mitogen in the rat (6).

From information available in the murine system we developed a blastogenic assay in the rat. In order to interface with our current standard
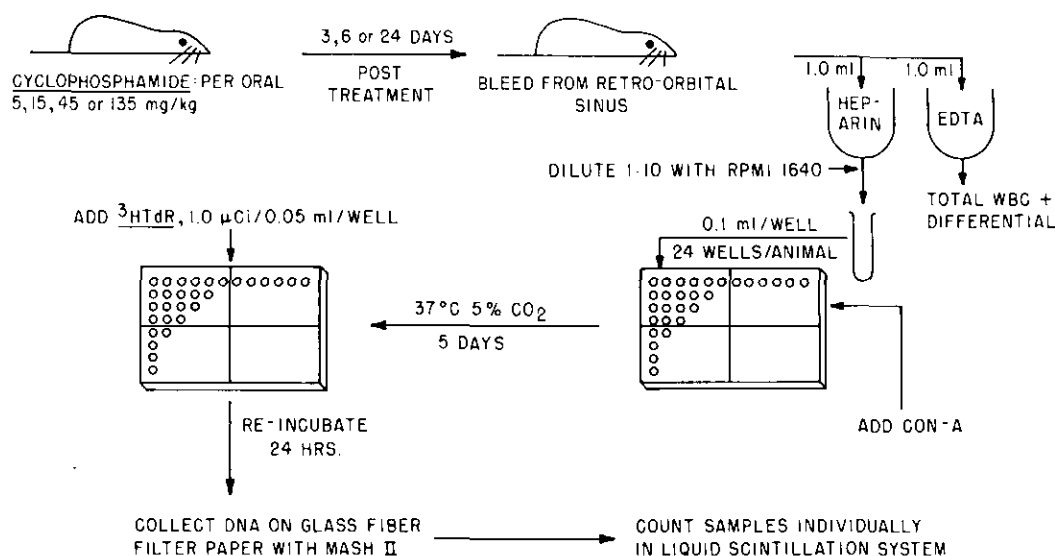
FIGURE 1. Protocol for the lymphoproliferative response of rat peripheral blood cells by mitogens.

operating procedures, we modified the whole blood assay described previously by Han and Pauly (7) (see Fig. 1). At the same time that the hemotologic sample was taken, a sample for the blastogenic assay was obtained. After a standard dilution, the cells were cultured with the appropriate mitogen and the functional activity of the respective populations of lymphocytes was assessed. During the course of development, we considered whether or not additional nutrients, such as fetal calf serum or

mercaptoethanol, should be used to supplement the media. The addition of these supplements did not increase the magnitude of the responsiveness sufficiently to warrant their use (Norbury, unpublished findings). In addition, it was reasoned that by adding them, any immunopotentiating effect might be masked.

With this technique we were able to show immunosuppressive activity with cyclophosphamide (8) and dexamethasone but not with aspirin (Table 1).

Table 1. Mitogen-induced proliferative responses of rat peripheral blood cells *in vitro* following various treatments *in vivo*

| Treatment group | Number of lymphocytes, % of control | Relative proliferation index[a] | | | | | |
| | | Concanavalin A | | Phytohemagglutinin | | Pokeweed mitogen | |
		(2-4 μg/well)	(0.32-0.64 μg/well)	(1.6-3.2 μg/well)	(6.4 μg/well)	(1.6-6.4 μg/well)	(1.6-6.4 μg/well)
Purification by dextran-citrate[b]		−	+	−	+	−	+
Cyclophosphamide (n = 8)[c]							
10 mg/kg	81	0.59*	3.29	0.44*	1.50	0.70	1.41
50 mg/kg	32	0.02*	0.70	0.04*	0.48*	0.13*	0.39
Dexamethasone (n = 8)[d]							
0.35 mg/kg	43	0.08*	1.48	0.05*	0.88	0.17*	1.03
0.70 mg/kg	41	0.01*	1.68	0.06*	0.99	0.15*	1.77
Aspirin (n = 5)[e]							
100 mg/kg/day	111	0.94	NT	NT	NT	NT	NT
200 mg/kg/day	123	0.84	1.96[f]	NT	NT	NT	NT

*Significantly different from controls ($p \leq 0.05$) using anti-rankit transformation.
[a]Relative proliferation index is calculated from the following formula: mean counts per minute (treated)/mean counts per minute (controls) at the optimal concentrations (indicated in parentheses) for each mitogen. NT = not tested.
[b]Lymphocytes were collected from the buffy coat and plated at a concentration of 10^5 cells per well in 96-well microtiter plates.
[c]Animals were given a single oral dose; peripheral blood was collected from the retro-orbital plexus 4 days later.
[d]Animals were given a single oral dose; blood was collected 2 days later.
[e]Animals were dosed orally for 30 days and then bled.
[f]Lymphocytes were depleted of erythrocytes by ammonium chloride lysis.

Environmental Health Perspectives

An appealing feature of this assay is the fact that an antigenic stimulus of the animal is not required, thus eliminating the need to manipulate animals in a manner that may jeopardize the integrity of the routine toxicological evaluation. However, one important disadvantage of this technique is not knowing the fate of the lymphocyte once it is cultured. In the case of cyclophosphamide, a slight reduction (19%) in the number of circulating lymphocytes caused a significant suppression of the lymphoproliferative response to both ConA and PHA. At higher doses, cyclophosphamide caused marked lymphopenia with concomitant reduction in the lymphoproliferative response. It was entirely possible that the blastogenic assay was merely a reflection of the number of immunocompetent cells in culture. However, when the number of lymphocytes separated by dextran-citrate was adjusted to a constant concentration based on viability, the residual lymphocytes from cyclophosphamide-treated animals still had significantly impaired responsiveness to PHA (Table 1). Better techniques will be needed to determine if the lymphocytes from treated animals are still viable after 5 days in culture, especially when using a whole blood preparation.

Enumeration of Lymphocyte Subpopulations

In the event that an immune function test demonstrates impairment, it behooves the investigator to determine the number of immunocompetent cells. Lymphocyte subpopulations can generally be classified into three broad categories: T-cells, B-cells, and null cells. It is now known, particularly with the T-cell population, that a variety of subsets (T helper, T suppressor, T killer, etc.) exists and the means to quantitate them needs to be seriously considered. Once again, various methods are available, including immunofluorescence, rosette formation, histochemistry, cell electrophoresis, cytolysis and fluorescence-activated cell sorting. Immunofluorescence is the most popular technique, especially in working with rodents. The rosette formation assay appears to have some limitations when working with species other than human and is prone to interlaboratory variation. Histochemical methods also have limited application aside from human studies and cell electrophoresis is too analytical for immediate use. The complement-dependent, antibody-mediated cytolytic test can be used for quantitating lymphocyte subpopulations of lymphocytes on a routine basis. (I am not implying that none of the other methods can or will eventually be modified for routine testing.) With commercially available rabbit anti-rat thymocyte serum plus complement we obtained approximately 90% lysis of rat thymocytes and 50-55% lysis of splenic lymphocytes using the Coulter counter. The application of this particular technique to other peripheral organ sources (lymph node and peripheral blood) has not been as fruitful. Obviously much more work is needed in this area.

From this three-part program, the concept of a

Table 2. Special follow-up studies using common immune models.

	Study
Cell-mediated immunity	Memory (secondary antigen exposure)
	Lymphocyte mediated cytotoxicity
	Mixed lymphocyte reaction
	Lymphokine production
Humoral immunity	Antibody response to T-independent antigen
	Memory
	Plaque-forming cell quantitation
Reticuloendothelial system	
Resident/recruited alveolar and peritoneal cells	Number
	Phagocytosis
	Response to lymphokines
	Cytotoxicity
	Chemotaxis
	Enzymatic activity
Polymorphonuclear leukocytes	Mast cell degranulation
	Chemotaxis
	Phagocytosis
Other	Immunopathology
	Stem cell colony formation
	Complement activity
	Antinuclear antibodies

basic screen evolved to provide a minimum number of assays that would allow a meaningful interpretation of immunologic alterations. The basic screen includes the determination of total serum IgG, IgM and IgA, blastogenesis with T- and B-cell mitogens as well as specific antigens, cell enumeration, total leukocyte and differential, delayed type hypersensitivity, primary antibody responses to a T-dependent antigen, clearance of particulate matter by the reticuloendothelial system, and histopathology. This satisfies most of our initial criteria for evaluating T-cell and B-cell responses as well as macrophages while keeping the number of assays to a minimum. Such assays are relatively simple to perform and the technology now exists to implement them. However, it does appear that we may have to include additional animals that would not necessarily be processed as part of the routine toxicity study.

Until disproven, my bias is that the evaluation of antibody titers to a T-dependent antigen will eventually represent the best single analysis, closely simulating the immune response to infection. An antibody assay is quite sensitive and represents a response to a defined antigen involving the same types of cells that mediate immunity to infection.

In the event that the basic screening reveals alterations or inadvertent modifications of the immune response, special follow-up studies (Table 2) can be carried out to clarify the mechanism of action and the cell types involved, with particular emphasis on defining the subpopulations affected by the drug. Nevertheless, it is important to understand the limitations of any assay before making a judgment regarding the effect of a drug on the immune response.

Future of Immunotoxicology

In addition to on-line testing, there are other areas where immunology can be applied within the pharmaceutical industry.

Skin Sensitization

New developments in the methodology used to evaluate drug-induced hypersensitivity, or skin sensitization, has made it possible to improve the credibility of certain obligatory tests. The Landsteiner-Draize guinea pig skin test has long been the method of choice for doing skin sensitization. However, the development of the skin maximization test (9) has greatly improved the predictability of the guinea pig model while eliminating false negatives.

Development of Biologicals

Some pharmaceutical companies are actively developing viral vaccines as well as other adjuvants. Immunotoxicology would offer a dimension beyond that capable of routine toxicology for the assessment of potential adverse effects that might be immunologically mediated.

Development of Specific Immune Modulators

While the search for new and better anti-inflammatory compounds is an active program of many institutions, the toxicological examination of these new product candidates, inclusive of immune function testing, offers a broader basis for determining the potential of that particular drug.

New Product Development

The toxicologic testing of compounds that are not specifically designed as immune modulators may, in remote instances, demonstrate immunologic enhancement or impairment. Such information could then be referred back to medicinal chemists and pharmacologists for further development.

Basic Research

To aid in the development of more rapid and reliable assays as well as new immune models, which are critical to understanding the effects of chemicals on the immune response, some effort should be made in an immunotoxicology program to conduct basic research.

Conclusion

The field of immunotoxicology is in the early stages of rapid development. Although the immune response is quite complex, involving a sophisticated array of cellular and molecular interactions, there is increasing evidence that chronic, subclinical exposure to a variety of chemicals does in fact lead to an adverse modification of immune function, manifested most ostensibly by reduced host resistance to infectious agents, such as viruses and bacteria (10). In many respects, the state-of-the-art is much like mutagenesis was five to ten years ago. Yet we must realize that the progress of immunotoxicology will depend upon the continued, close interaction among persons in academia, industry, as well as governmental institutions. With the approach evolving at MSDRL, it now seems possible to move ahead in this area. Ultimately, a basic screen for

immune competence needs to be validated using compounds with known immunosuppressive effects as part of a subacute study. In doing so, one must be prepared to explain any alteration in immune function in the absence of overt toxicity as typically described by physical observation, hematological and serum biochemical studies, urinalyses, and gross and histomorphologic examination. With further development, immunotoxicology can become part of the repertoire of toxicity testing, as we have seen for teratology and mutagenicity.

REFERENCES

1. Vos, J. G. Immune suppression as related to toxicology. CRC Crit. Rev. Toxicol. 5: 67-101 (1977).
2. Makinodan, T., and Adler, W. H. Effects of aging on the differentiation and proliferative potential of cells of the immune system. Fed. Proc. 34: 153-158 (1975).
3. Buskirk, H. H., Crim, J. A., Petering, H. G., Merritt, K., and Johnson, A. G. Effect of uracil mustard and several antitumor drugs on the primary antibody response in rats and mice. J. Natl. Cancer Inst. 34: 747-758 (1965).
4. McDevitt, H. O. and Landy, M. Genetic Control of Immune Responsiveness, Academic Press, New York, 1972.
5. Ritchie, R. F. Specific protein assay. Fed. Proc. 34: 2139-2144 (1975).
6. Naot, Y., Merchar, S., and Ginsburg, H. *Mycoplasma neurolyticum*: A potent mitogen for rat B lymphocytes. Eur. J. Immunol. 9: 185-189 (1979).
7. Han, T., and Pauly, J. Simplified whole blood method for evaluating *in vitro* lymphocyte reactivity of laboratory animals. Clin. Exptl. Immunol. 11: 137-142 (1972).
8. Norbury, K. C. and Noble, C. Routine assessment of immunocompetence in rats: The effect of cyclophosphamide. In: Inadvertent Modification of the Immune Response: The Effects of Foods, Drugs, and Environmental Contaminants, I. M. Asher, Ed., The Office of Health Affairs, FDA, Washington, D.C., 1978.
9. Magnusson, B., and Kligman, A. M. The identification of contact allergens by animal assay. The guinea pig maximization test. J. Invest. Dermatol. 52: 268-276 (1969).
10. Koller, L. D. Effects of environmental contaminants on the immune system. Adv. Vet. Sci. Comp. Med. 23: 267-295 (1979).